Evidence of Nuclear PKC/MAP-Kinase Cascade in Guinea Pig Model of Epidermal Hyperproliferation

Indu Mani, Lars Iversen,* and Vincent A. Ziboh

Department of Dermatology, University of California, Davis, California, U.S.A.; *Department of Dermatology, Marselisborg Hospital, University of Aarhus, Aarhus, Denmark

In order to delineate the biochemical events in the nuclear compartment of an *in vivo* proliferating epidermis, we produced a model of hyperproliferative epidermis by topical application of docosahexaenoic acid (22:6n-3) on guinea pig skin. Employing this model we demonstrated: (i) that protein kinase C (PKC)-α and atypical PKC-ζ are the two major PKC isozymes in the normal epidermal nuclear membrane, in contrast to PKC-α and PKC-β in the epidermal plasma membrane; (ii) that topical application of docosahexaenoic acid induced epidermal hyperproliferation and enhanced total nuclear PKC, particularly nuclear PKC-α and the atypical PKC-ζ isozymes. The increase in the nuclear PKC isozymes paralleled a marked increase in the expression of nuclear mitogen-activated protein-kinase. These data suggest that epidermal hyperproliferative activity is accompanied by the upregulation of nuclear PKC/mitogen-activated protein-kinase signaling pathway. ***Key words: . J Invest Dermatol 112:42–48, 1999***

Linoleic acid (LA, 18:2n-6) is the most abundant polyunsaturated fatty acid in the human skin (Chapkin *et al*, 1987). The exclusion of LA from the diet induces a defect in the skin that is characterized by epidermal hyperproliferation that is reversible by inclusion of LA in the diet. Hence, LA was heralded as an "essential fatty acid (EFA)" (Burr and Burr, 1930). The recognition that LA is essential in maintaining normal skin prompted extensive study in order to delineate the link between cellular proliferation and LA in the epidermis. The possibility that an oxidative metabolite of LA may be involved in catalyzing the reversal of cutaneous hyperproliferation was first suggested by Nugteren *et al* (1985); although the nature of this metabolite and its mechanism of action remained unknown. In extending the preceding observation, we demonstrated that LA was metabolized by normal human or guinea pig epidermal 15-lipoxygenase, mainly to 13-hydroxyoctadecadienoic acid (13-HODE), and that the level was high in normal epidermis. In contrast, the level of 13-HODE in the EFA hyperproliferative guinea pig epidermis was markedly depressed (Cho and Ziboh, 1995), suggesting that altered epidermal 13-HODE may be associated with epidermal hyperproliferation.

Protein kinase C (PKC) comprises a family of ser/thr kinases that have been divided into three distinct groups: the conventional, calcium, phospholipid, and diacylglycerol-dependent PKC (PKC-α, -β, -γ), the novel, calcium-independent PKC (nPKC-δ, -ε, -η, -θ), and the atypical PKC (aPKC-ζ, -ι, -λ), which are also calcium, phospholipid, and diacylglycerol-independent (reviewed in Nishisuka, 1992; Hug and Sarre, 1993). These isozymes are expressed in a cell and tissue-specific manner and appear to be involved in cell proliferation and differentiation (Berra *et al*, 1993; Osada *et al*, 1993; Dlugosz *et al*, 1994; Whitfield *et al*, 1996). The importance of PKC in the epidermis was first suggested when the tumor promoting phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, a direct activator of PKC, induced both differentiation and proliferation in the skin (Griffiths *et al*, 1988; LePanse *et al*, 1994). A number of subsequent studies have demonstrated the presence of multiple PKC isozymes in the epidermis (Wang *et al*, 1993; Fisher *et al*, 1993). It has also been reported that the subcellular localization and functions of these isozymes vary depending on the species and the type of cells studied (Gherzi *et al*, 1992; Cho and Ziboh, 1994). In human epidermis, PKC-β has been reported to predominate in the Langerhans cells, whereas the atypical aPKC-ζ has only been reported in keratinocytes (Fisher *et al*, 1993). Using the guinea pig epidermis as an *in vivo* model, we previously demonstrated that the prominent isozymes in the cytosolic and microsomal fractions are PKC-α and -β (Cho and Ziboh, 1994a).

Because cell growth and proliferation are regulated by biochemical events in the nuclear compartment of the cell, it was essential to establish an *in vivo* model of epidermal hyperproliferation in order to delineate the nuclear biochemical events that take place during the hyperproliferative process. In a previous report from this laboratory, we demonstrated that topical application of the long chain n-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA, 22:6 n-3) on the skin of guinea pigs induced an acute hyperproliferation of the epidermis (Miller and Ziboh, 1990). The morphologic alteration was accompanied by a marked decrease in the epidermal level of 13-HODE, a 15-lipoxygenase metabolite of LA. These morphologic and lipid alterations paralleled a marked increase in the membrane-associated PKC-α and PKC-β activities (Cho and Ziboh, 1994b). Because nuclear PKC activation has been implicated with the ability of the cell to respond to mitogenic signals such as growth factors (Fields *et al*, 1989), it was of interest

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Reprint requests to: Dr. V.A. Ziboh, Department of Dermatology, TB 192, University of California at Davis, Davis, CA 95616.

Abbreviations: aPKC, atypical PKC; DHA, docosahexaenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 13-HODE, 13-hydroxyoctadecadienoic acid; LA, linoleic acid; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase-kinase; nPKC, nuclear PKC; PKC, protein kinase C; PUFA, polyunsaturated fatty acid.

to investigate the effects of DHA-induced hyperproliferation on nuclear biochemical processes. We reason that the activation of nuclear isoforms of PKC would implicate them in the regulation of nuclear signaling events. Consistent with this view, there is now increasing evidence indicating that PKC is associated with the nuclear events of different tissues, both in the resting as well as in the activated cells (Capitani *et al*, 1987; Chiarugi *et al*, 1990; Buchner *et al*, 1992; Neri *et al*, 1994). It is also now evident that, in addition to phosphorylation and activation of various cytoskeletal intracellular proteins, PKC is involved in several nuclear signal transducing events (Buchner *et al*, 1992; Rosenberger *et al*, 1995). Activation and nuclear translocation of PKC isozymes have been reported in Swiss 3T3 cells, NIH fibroblasts, and rat liver heptatocytes (Chiarugi *et al*, 1990; Neri *et al*, 1994). The atypical PKC-ζ has been reported as critical for the activation of NK-κβ (Lozano *et al*, 1994) and for mitogenic signaling in mammalian cells and xenopus oocytes (Berra *et al*, 1993).

Another set of protein kinases that play an important role in transducing signals that originate at the cell membrane to the nucleus are the mitogen-activated protein (MAP) kinases (Arenzana-Seididos *et al*, 1989; Nishida and Gotoh, 1993; Berra *et al*, 1995). These MAP-kinases are activated by combined tyrosine and threonine phosphorylation catalyzed by MAP-kinase-kinase (MEK), a novel class of protein kinases with dual specificity for both tyrosine and serine/threonine. One nuclear target of these MAP-kinase signaling pathways is the transcription factor AP-1 (Bernstein *et al*, 1994). They have also been shown to be involved with the activation of the transcription factor NF-κβ (Berra *et al*, 1995).

To delineate whether a relationship exists between DHA-induced epidermal hyperproliferation and nuclear PKC/MAP-kinase signaling cascade, we determined as a first step the types of PKC isozymes that are endogenous in the nuclear fraction of the normal guinea pig epidermis. Next we tested whether or not these nPKC were altered in the DHA-induced hyperproliferative epidermal model. Because the MAP-kinase signaling pathway functions to transduce information from the plasma membrane to the nucleus, we determined whether or not alterations do occur in the expression of two members of the MAP-kinase cascade: the MEK and the p42/p44 MAP-kinase in the epidermal nuclear membrane preparations from normal and DHA-induced hyperproliferative skin epidermis.

MATERIALS AND METHODS

Animal treatment To produce an acute guinea pig model of epidermal hyperproliferation, male Hartley guinea pigs (450–475 g) were purchased from Simonsen Laboratory (Gilroy, CA). The hair from the dorsal skin was shaved and depilated with sodium thioglycollate (Nair, Carter Wallace, New York) and then demarcated into two regions along the spine. The guinea pigs were then treated on the left side with vehicle/control [ethanol/propylene glycol (70:30), containing 1% vitamin E] and on the right side with vehicle-containing DHA (2%). The treatments were applied topically, twice daily (1.25 ml per side) covering an area of ≈4 × 12 cm. In order to discern the time course of the development of the acute epidermal inflammation and/or hyperproliferation, animals were sacrificed after 2, 6, 24, 48, and 96 h, respectively. One portion of the epidermis was removed for histologic evaluation and another used for the determination of DNA synthesis. To determine the fate of the topically applied DHA, another portion of the epidermis was homogenized and used to determine the incorporation of DHA into epidermal phospholipids as well, for the quantitation of endogenous levels of the epidermal hydroxy fatty acids. The major portion of the epidermis was used for the isolation of epidermal nuclear particulate that was used for PKC and MAP-kinase determinations.

Histologic evaluation and epidermal DNA synthesis To compare the histology of the two experimental groups – the vehicle/control and the DHA-treated skin – 4 mm biopsies from the dorsal skin were obtained and placed in neutral buffered Formalin. These specimens were fixed, stained with hematoxylin and eosin, sectioned to a thickness of 6 μm, and affixed to slides for viewing by light microscopy (Histo Path, Modesto, CA) as previously described (Miller and Ziboh, 1990).

Epidermal DNA synthesis To establish the degree of tissue hyperproliferation, epidermal discs (4 mm) were incubated in 5 ml of Dulbecco's modified Eagle's medium containing 1 μCi[^{3}H]thymidine per ml at 37°C for 3 h. The discs were then washed in phosphate-buffered saline to remove adherent [^{3}H]thymidine, resuspended in 1 ml of 0.5 N NaOH, and placed in a boiling water bath for 30 min to dissolve the tissue and release DNA. An aliquot of this solution was placed on Whatman filter paper discs that were previously treated with 10% trichloroacetic acid to aggregate DNA-associated proteins. The discs were dried at 110°C, placed in scintillation vials containing 5 ml of scintillation fluid, and counted. Another aliquot was used for measuring endogenous DNA content (Labarca and Paigen, 1980). The rate of DNA synthesis was expressed as [^{3}H]thymidine incorporated per μg DNA.

Time course of DHA-induced hyperproliferative skin Because the development of skin inflammatory/hyperproliferative lesion was time dependent, we determined the time course of DHA-induced epidermal hyperproliferation. To accomplish this, guinea pigs were treated twice daily for 4 d with either vehicle alone or containing DHA (2%) (2.5 ml per animal) and covering an area of ≈8 × 12 cm as described previously (Miller and Ziboh, 1990). Specifically, the dorsal skin was demarcated into two regions along the spine. The left side was treated with 2% DHA contained in vehicle, whereas the right side was treated with vehicle alone (1.25 ml per side). Groups of animals were sacrificed at 2, 6, 24, 48, and 96 h, respectively. The epidermis at each time point was removed and evaluated for histology, for tissue phospholipids, hydroxy fatty acids, and for tissue expression of nuclear PKC and MAP-kinase.

Analyses of epidermal phospholipid fatty acids and hydroxy fatty acids The epidermal slices removed after the various time intervals were separately minced, homogenized in Buffer A [50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2.5 mM KCl, 1 mM ethylenediamine tetraacetic acid, 0.5 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 1 mg leupeptin per liter, 1 mg aprotinin per liter] containing 0.33 M sucrose and then filtered through cheese cloth. A portion of the clear homogenate was acidified to pH 3.0, centrifuged to remove debris and the supernatant applied to the preconditioned octadecylsilyl (ODS) C_{18} silica column (Sep-pak silica cartridge; Waters Chromatography Division, Milford, MA). The polar phospholipid fraction was first eluted from the column with 25% MeOH in water followed by elution of the prostaglandins/leukotrienes/hydroxy fatty acids with 100% MeOH.

Epidermal total phospholipid fatty acids The phospholipid fraction eluted from the Sep-pak column at each time point was dried in a rotavap and the resulting residue transmethylated in 6 ml of 6% methanolic HCl. As reported previously, the resulting fatty acid methyl esters were separated and quantitated in a Shimadzu model GC-17A, gas chromatography (Pleasant, CA) equipped with a DB-225 used silica capillary column (50% cyanopropylphenyl, 0.15 mm film thickness) 30 m × 0.25 mm i.d. (J&W Scientific, Rancho Cordova, CA). Hydrogen (36 cm per s) was used as the carrier gas. The oven was run isothermally at 200°C, and detection of the fatty acid methyl esters was performed with a flame ionization detector. An internal standard, heptadecaenoate, was added for quantitation.

Epidermal hydroxy fatty acids The MeOH fraction from the Sep-pak column at each time point was dried under N_2 gas and then resuspended in ethanol. An aliquot from each ethanol extract was injected into the Beckman Ultrasphere 5 μm ODS (RP-HPLC) column as previously described (Miller *et al*, 1991). Briefly, the mobile phase, consisting of methanol/water/acetic acid (70:30:0.01) by volume, was run first at a flow rate of 0.8 ml per min for 0–10 min, and then increased to 1.8 ml per min for 10–60 min. The separated lipoxygenase products were monitored by characteristic wavelength absorbances. The hydroxy fatty acids were monitored at 234 nm and identified by chromatographic comparison with authentic standards. Results were expressed as ng hydroxy fatty acid per mg protein in the crude epidermal homogenate.

Isolation of epidermal nuclear fraction Epidermal nuclear fraction was prepared as previously reported by Blobel and Potter (1996) with minor modifications. Briefly, epidermis was minced and homogenized in Buffer A containing 0.33 M sucrose. The homogenate was filtered through cheese cloth and centrifuged at 800 × *g* for 12 min. The resulting pellet was resuspended in Buffer A containing 0.25 M sucrose. Two volumes of Buffer A containing 2.3 M sucrose were added to bring the sucrose concentration to 1.62 M. This suspension was layered over 1 volume of

Buffer A containing 2.3 M sucrose and centrifuged at 100,000 × *g* for 45 min. The pellet at the bottom of the sucrose cushion was washed twice by resuspension in Buffer A containing 0.25 M sucrose using a Dounce homogenizer and centrifugation at 800 × *g* for 12 min. The nuclear pellet was resuspended in a lysis buffer containing 5 mM dithiothreitol and 1% Triton X-100. The purity of the nuclear fraction was confirmed indirectly by assaying for the activity of 5′ nucleotidase (Kai *et al*, 1966) as the marker for plasma membrane, thus excluding plasma membrane contamination of the nuclear fraction. Another aliquot was used to determine the activity of lactate dehydrogenase (Storrie and Madden, 1990) as the cytosolic marker, thus excluding cytosolic contamination. The activities of both enzymes in the nuclear fraction were less than 1% of their activities when compared with activities of these enzymes in the other subcellular compartments. The localization of these enzymes in the three subcellular fractions are shown in **Table I.**

Assay of total nuclear PKC activity Assays to determine total nuclear PKC activity in the purified nuclear fraction were carried out as described previously (Cho and Ziboh, 1994a). Briefly, the reaction was initiated by the addition of 5 μg of nuclear protein to a 250 μl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2 g phosphatidylserine per liter, 4 μM 1,2-dioleylglycerol (diolein), 50 mg Histone (III-S) substrate per liter, and 10 μM [γ^{32}P]ATP (5 × 10^6 dpm per nmol). Phosphatidylserine and diolein were suspended in 0.3% Triton X-100 before addition to the assay mixture. Incubations were carried out for 10 min at 30°C and then terminated by the addition of 2 ml of ice cold 25% trichloroacetic acid. The precipitated labeled proteins were collected on a Millipore HA filter (0.45 μm). Each filter was washed eight times with 3 ml each of 5% trichloroacetic acid and the radioactivity counted by liquid scintillation in a Beckman LS 3501 counter using Beckman Ready Gel. Control experiments were carried out without the nuclear extracts, or with heat-inactivated nuclear protein extracts. The nonspecific radioactivity from the control experiments was subtracted from those with nuclear extracts before estimating enzyme activity. The enzyme activity was expressed as ^{32}P incorporated (cpm) per mg protein. The protein concentration was determined using the Bio-Rad Bradford reagent. To determine the activity of the aPKC, the assay was carried out in the absence of $CaCl_2$, phosphatidylserine, and diolein, but with 2 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

Identification of nuclear PKC isozymes by western blot assay A portion of the nuclear fraction (10 μg protein) from either normal epidermis or DHA-treated epidermis was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5%). The use of equal amounts of nuclear fraction afforded uniformity of protein samples from each preparation. The resolved proteins were transferred to PVDF membranes and the gel stained to verify equal loading and uniform transfer of the proteins. Nonspecific sites on the membrane were blocked by incubation with 10% nonfat dry milk overnight at 4°C. Western blot assay was performed using antibodies against the various isozymes of PKC (α, βI, βII, γ, δ, ε, ζ, η) (GIBCO BRL, Grand Island, NY). The membranes were then washed and incubated with a biotinylated secondary antibody (rabbit anti-goat IgG) (Vector, Burlingame, CA) at room temperature for 1 h. This was followed by washing and incubation with the vectastain ABC reagent (Vector) for 1 h. The bands were visualized by enhanced chemiluminescence using the ECL kit (Amersham).

Table I. Subcellular enzyme markers[a]

	Marker enzymes	
Subcellular fractions	5′ Nucleotidase (μg p per mg protein)	Lactic dehydrogenase (LDH) (Absorbance per min per mg protein)
Cytosol	nd	1.63 ± 0.15
Microsomal (membrane)	3.4 ± 1.12	0.45 ± 0.08
Nuclear (membrane)	nd	0.041 ± 0.001

[a]Epidermal slices were taken from normal guinea pig skin, homogenized and subjected to subcellular centrifugation into nuclear, cytosolic, and microsomal fractions as detailed under *Materials and Methods*. Aliquots from each subfraction were analyzed for activities of 5′ nucleotidase (as plasma membrane marker) and lactate dehydrogenase (as cytosolic marker). Data represent means ± SEM of four separate experiments. nd, not detected; 5′ nucleotidase, plasma and microsomal membrane marker; LDH, cytosolic marker.

Expression of nuclear MEK and MAP-kinase by western blotting In addition to identification of PKC isozymes, aliquots of the nuclear protein were also probed, respectively, with antibodies to p42/p44 MAP-kinase as well as the antibody to MEK (Sigma, St. Louis, MO). Incubation with secondary antibodies and detection of bands were the same as for the PKC isozymes. The nuclear membranes were next scanned using a UMAX S-6E scanner and densitometric analysis of the bands were carried out using the Bio Image Intelligent Quantifier software (B.I. Systems, Fremont, CA)

Statistical analysis All results were subjected to one-way analysis of variance (ANOVA). For comparison between the observations, the two-tailed t test was used. The probability (p) of statistical significance was at 0.05 or below. The values in the text are means ± SE.

RESULTS

Histologic evaluation of DHA-induced hyperproliferation in normal guinea pig skin Histologic evaluations of vehicle-treated and DHA-treated epidermis at varying time periods are shown in **Fig 1**. Epidermal thickening and hyperkeratosis were

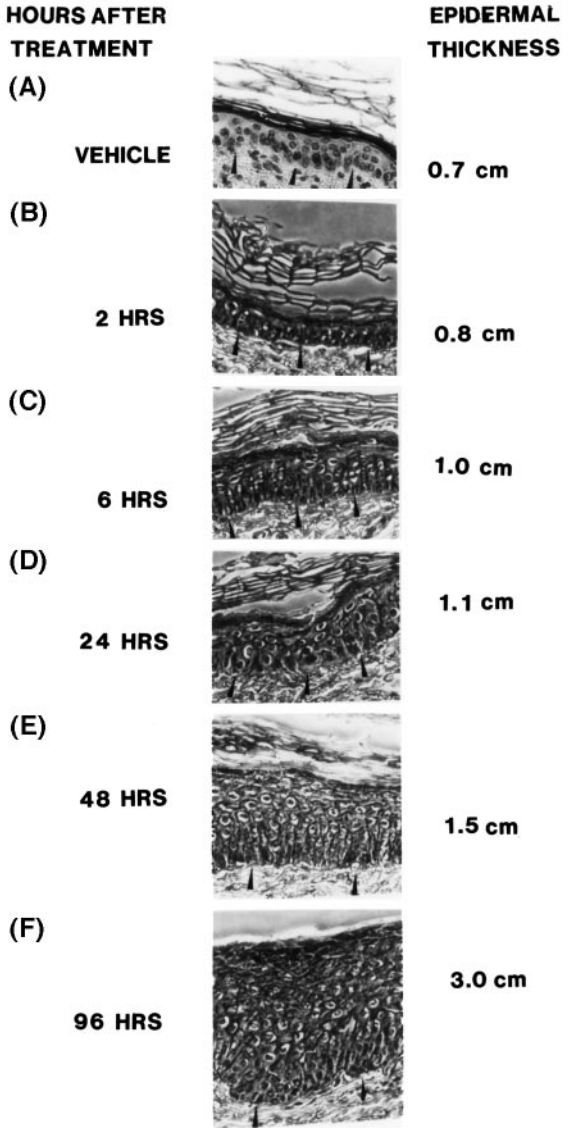

Figure 1. Histologic evaluation of DHA-induced hyperproliferation in normal guinea pig skin (time course development) (magnification ×200). Skin in (*A*) was treated with vehicle [ethanol/propylene glycol (30:70) containing 1% vitamin E as antioxidant]. The pictures in (*B*)–(*F*) (2–96 h) represent skin treated with 0.5% DHA (in vehicle containing 1% vitamin E). Histologic evaluations were stained with hematoxylin and eosin. Note that epidermal thickness from 24 h to 96 h specimens revealed increasing epidermal thickness

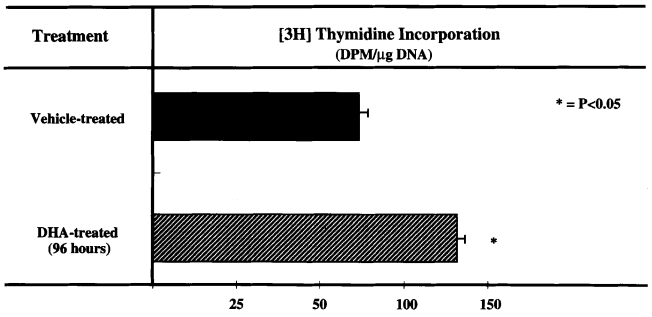

Figure 2. [^{3}H]Thymidine incorporation into epidermal DNA in vehicle-treated (normal) and DHA-treated (hyperproliferative) guinea pig skin. To measure the degree of DNA synthesis, epidermal discs (4 mm^2) from vehicle- and DHA-treated skin were incubated with [^{3}H]thymidine as described under *Materials and Methods*. DNA level was analyzed using the fluorescent reaction with bisbenzimide (H33258). Values are presented as dpm per μg DNA and represent means ± SEM (n = 6) from three separate experiments.

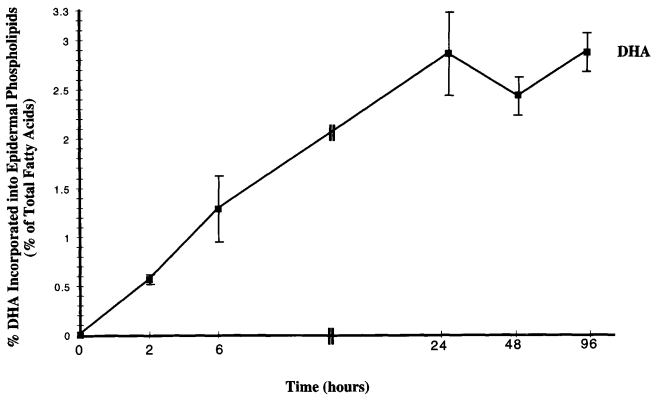

Figure 3. Incorporation of topical DHA into epidermal total phospholipids. Time course of the incorporation of DHA into epidermal phospholipids was determined at 2, 6, 24, 48, and 96 h, respectively. Briefly, the epidermis was homogenized, acidified to pH 3.0, and passed through octadecylsilyl C_{18} silica column (Sep-Pak) as detailed in *Materials and Methods*. The eluted polar fraction (containing phospholipids) was subjected to methanolic-HCl to obtain fatty acid methyl esters. The methyl esters were separated by gas liquid chromatography as detailed under *Materials and Methods*. The amount of DHA in total phospholipid at each time point is expressed as the percentage of DHA in total phospholipid fatty acids.

minimal during the first 6 h after treatment with DHA; however, DHA treatment after 24 h (day 1), 48 h (day 2), and 96 h (day 4) revealed evidence of marked epidermal hyperproliferation that was characterized by acanthosis (thickening of the spinous layer), hypergranulosis (thickening of the granular layer), and hyperkeratosis (thickening of the stratum corneum) when compared with vehicle-treated skin. The changes in histologic appearance of the epidermis paralleled the increased uptake of [^{3}H]thymidine. For instance, the DHA-treated epidermis at 96 h showed marked increase in [^{3}H]thymidine uptake when compared with vehicle-treated control **Fig 2**.

Topical DHA treatment enhances DHA incorporation into epidermal total phospholipids In order for DHA to exert any recognizable effect on skin epidermis, we reasoned that DHA has to be incorporated into epidermal phospholipids. This view is consistent with a previous study from this laboratory in which dietary feeding of guinea pigs with a diet supplemented with fish oil (containing EPA and DHA) resulted in the incorporation of both EPA and DHA into epidermal phospholipids (Miller *et al*, 1991). As shown in **Fig 3**, the incorporation of DHA into epidermal total phospholipids was time dependent and incorporation was maximal after 24 h and remained high at 96 h.

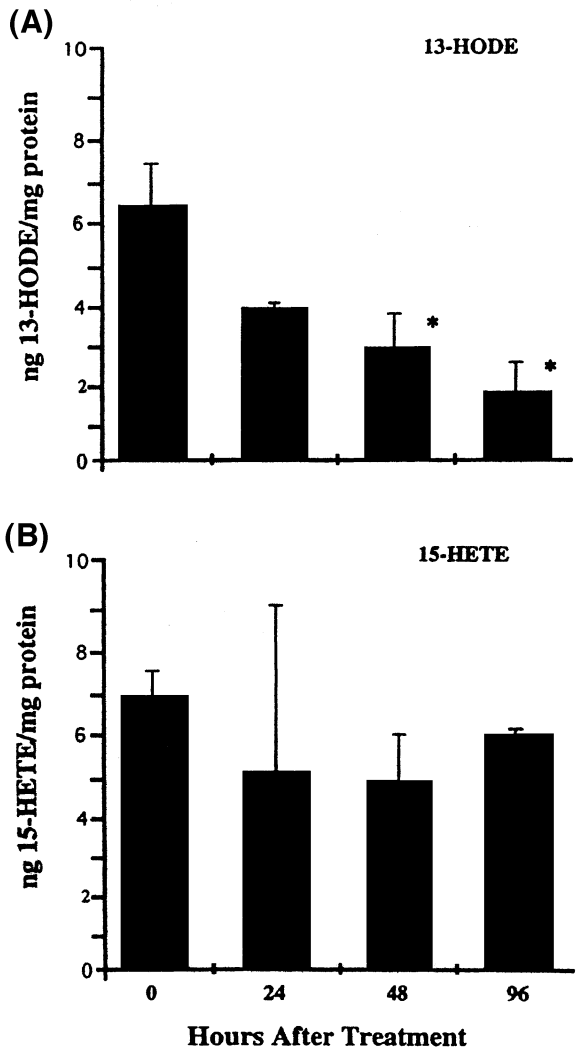

Figure 4. Time course of DHA effects on epidermal hydroxy fatty acids. Effect of (*A*) 13-HODE and (*B*) 15-HETE. To determine epidermal levels of hydroxy acids, epidermal strips taken from DHA-treated and vehicle-treated skin were homogenized and passed through the Sep-pak as indicated in **Fig 3**. The eluted methanol extract (containing hydroxy acids) were subjected to separation on RP-HPLC as detailed under *Materials and Methods*. The value at each time point is expressed as ng epidermal per mg protein. Each point represents means ± SEM of three separate experiments. *$p < 0.05$.

Topical treatment of DHA suppresses endogenous level of epidermal 13-HODE Because of the presence of an active epidermal 15-lipoxygenase in both guinea pig and human epidermis, we determined whether or not topical DHA would exert any effect on the two important 15-lipoxygenase products, 13-HODE (derived from linoleic acid) and 15-HETE (derived from arachidonic acid), in the epidermis. The data in **Fig 4**(***A***) revealed that DHA treatment moderately suppressed 13-HODE (38%) during the first initial 24 h; however, significant suppression of epidermal 13-HODE was evident after 24 h, and at 48 h and 96 h were 57% and 77%, respectively, when compared with epidermis from vehicle-treated skin. In contrast, the epidermal levels of 15-HETE (the 15-lipoxygenase product derived from arachidonic acid) were only moderately altered (**Fig 4*B***). The reason and significance of this differential generation of 13-HODE and 15-HETE are unknown. It is interesting to note that two isoforms of 15-lipoxygenase have been described: one of which was selective for linoleic acid and the other for arachidonic acid. It is unclear from these experiments whether or not the effect of DHA was selective on either of these isoforms of 15-lipoxygenase. Alteration of 13-HODE none the less

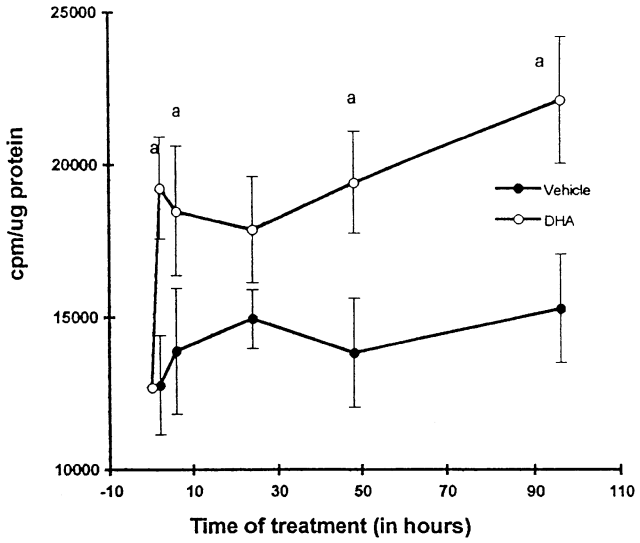

Figure 5. Time course of topical DHA treatment on total nuclear PKC activity in epidermal nuclear fraction. To determine the activity of total nuclear PKC at each experimental time point, an aliquot of the purified nuclear protein (5 μg) was added to a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2 g phosphatidylserine per liter, 4 (M1,2-dioleylglycerol (diolein), 50 mg histone (III-S) substrate per liter, and 10 μM [γ^{32}P] ATP (5 kBq per nmol). Phosphatidylserine and diolein were suspended in 0.3% Triton X-100 before addition to the assay mixture. Incubations and determination are as detailed in the *Materials and Methods*. The enzyme activity at each time point is expressed as ^{32}P incorporated (cpm) per μg protein and represents mean ± SEM from three separate experiments. The protein concentration was determined using the Bio-Rad Bradford reagent. The letter "a" in represents significant difference ($p < 0.05$) determinations at 2 h, 6 h, 48 h, and 96 h, respectively.

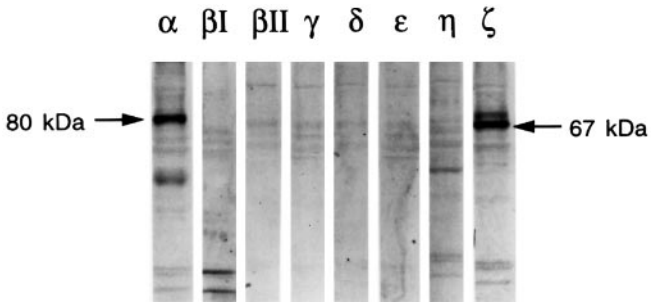

Figure 6. Identification of PKC isozymes in the nuclear fraction of guinea pig epidermis. To ascertain the PKC isozymes in the epidermal nuclear compartment a portion of the purified epidermal nuclear fraction (10 μg) was subjected to SDS-PAGE on a 7.5% gel followed by western blot assay with the different isozymes of PKC as detailed under *Materials and Methods*. The figure is a representative of results obtained from four different guinea pigs.

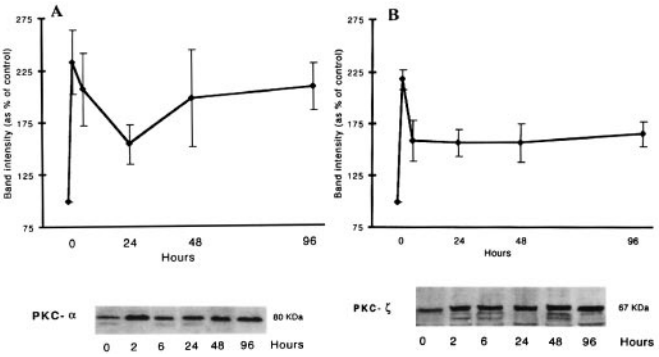

Figure 7. Time dependent alteration of epidermal nuclear PKC-α (*A*) and aPKC-ζ (*B*) isozymes by topical DHA. To ascertain the time-dependent alteration of PKC-α and aPKC-ζ, a portion of the purified epidermal nuclear fraction (10 μg) from vehicle-control and DHA-treated epidermis at each experimental time point was subjected to SDS-PAGE on 7.5% gel followed by western blot assay using antibodies against PKC-α and aPKC-ζ isozymes. The figure is representative of the densitometric analysis of the bands at each time point. Each point represents means ± SEM of four separate experiments.

underscored the significance of 13-HODE in maintaining normal epidermal homeostasis. Interestingly, the marked suppression of 13-HODE at 48 h and 96 h (**Fig 4*A***) paralleled the significant incorporation of DHA into the epidermal total phospholipids as shown in **Fig 3**.

Time-dependent upregulation of nuclear total PKC activity by topical DHA To discern whether or not nuclear total PKC activity was altered after topical DHA, a portion of the purified nuclear fraction at each time point was removed and the total PKC activity was assayed as described in the *Materials and Methods*. The data in **Fig 5** revealed that topical DHA induced a rapid increase in total nuclear PKC activity at 2 h, which then levelled out until 24 h. A second sustained increase emerged at 48 h and remained elevated until 96 h. Epidermis from vehicle-treated control animals remained relatively unchanged from 6 to 96 h.

PKC-α and PKC-ζ are major PKC isozymes in epidermal nuclear fraction Previous reports from our laboratory had demonstrated that topical application of DHA to guinea pig epidermis did induce hyperproliferation and increased DNA synthesis in the tissue. Furthermore, these alterations were accompanied by increased expressions and activities of plasma membrane-bound PKC-α and PKC-β isozymes (Cho and Ziboh, 1994a). These observations prompted us to discern whether or not similar biochemical events do occur in the nuclear compartment. Because cell growth and proliferation are controlled by nuclear biochemical events, we investigated whether or not nuclear PKC isotypes were also altered during DHA-induced epidermal hyperproliferation. To determine the profile of the nPKC isozymes expressed in the nucleus, a portion of the purified nuclear fraction was subjected to SDS-PAGE/western blot analysis using antibodies against individual isozymes. The data shown in **Fig 6** revealed that of all the nPKC isozymes tested, positive bands were clearly obtained only with the PKC-α and the aPKC-ζ isozymes. Thus, these two PKC isozymes are seemingly the constitutive, basal nPKC isozymes associated with guinea epidermal nuclear membrane. The nPKC-α was seen as a single band at 80 kDa whereas the aPKC-ζ appeared as a doublet-protein band with the stronger band appearing at 67 kDa. The reason for this repeated appearance of the doublet band in the guinea pig epidermis is unknown; however, the PKC-ζ has been reported as a doublet-protein band in mouse epidermal cells (Nishikawa *et al*, 1995). The significance of this doublet protein band is unknown. The properties and significance of this doublet band was not further pursued in this study. It is interesting that PKC-β that was identified as a major PKC isozyme in the guinea pig epidermal plasma membrane fraction was not detected in our purified nuclear fraction.

Time-dependent upregulation of epidermal nuclear PKC-α and aPKC-ζ isozymes by topical DHA The electrophoretic profile in **Fig 7**(***A***, ***B***) demonstrated the time-dependent upregulation of both nuclear PKC isozymes: nPKC-α and aPKC-ζ after DHA-treatment of the epidermis. Interestingly, increased expression of the nuclear PKC isozymes was initially rapid, decreased within the first 24 h, but remained significantly elevated ($p < 0.05$) at 48 h and 96 h. Furthermore, the time course of the upregulation of the nuclear total PKC isozymes paralleled the histologic evidence of hyperproliferation revealed in **Fig 1**. The upregulation of the nuclear PKC isozymes during the phase of epidermal hyperproliferation is consistent with a previous observation in the plasma membrane (Cho and Ziboh, 1994a).

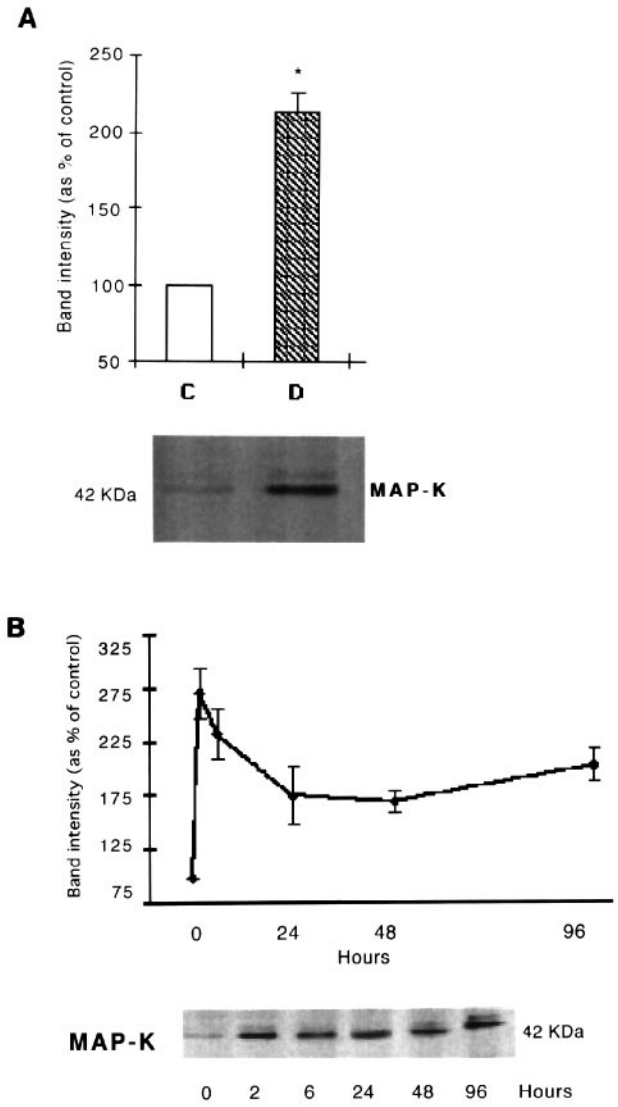

Figure 8. Identification and time course of the expression of MAP-kinase in the guinea pig epidermal nuclear fraction. A portion of the purified nuclear fraction (10 μg) was subjected to SDS-PAGE on a 10% gel followed by western blot analyses using two anti-MAP-kinase antibodies The bands were detected by chemiluminescence and also scanned using a UMAX S-6E scanner. The densitometric analysis of the bands was carried out using the Bio Image Intelligent Quantifier software as detailed under *Materials and Methods*. Part (*A*) illustrates the identification of MAP-kinase in vehicle (C) and DHA (D)-enhancement at 96 h. Each point as shown in (*B*) represents results obtained from four different guinea pigs.

Topical DHA upregulates expression of MAP-kinase in the epidermal nuclear fraction The electrophoretic evaluation as shown in **Fig 8**(***A***) revealed a detectable expression of basal MAP-kinase in the normal epidermal nuclear fraction. It appears that the p42/p44 MAP-kinases are the prominent members of the ERK (extracellular signal-regulated kinase) family of proteins in the nuclear membrane.

The treatment of skin with DHA caused an upregulation of MAP-kinase expression ($p < 0.05$) at 96 h as indicated in **Fig 8**(***A***) by the densitometric band intensity when compared with vehicle treated control. When the time course of the MAP-kinase expression was evaluated, the MAP-kinase expression revealed a time-dependent induced expression of MAP-kinase as shown in the data (**Fig 8*B***). The induction of MAP-kinase was rapid followed by a significant decrease at 24 h. The MAP-kinase expression none the less remained above control until 96 h. The level of the MEK was too low for the detection in our purified nuclear preparations.

DISCUSSION

Data from these studies provide a fitting experimental animal model for investigating the mechanism(s) of chemically induced epidermal hyperproliferation. It should be noted, however, that DHA is a dietary n-3 polyunsaturated fatty acid derived from fish oil. The data also explains, at least in part, why the dietary supplementation of EFA-deficient guinea pigs with fish oil failed to reverse the cutaneous hyperproliferative symptoms induced by EFA deficiency (Chapkin *et al*, 1987). One possibility why topical DHA did induce epidermal hyperproliferation is due at least in part to its inhibition of 13-HODE biosynthesis from LA (an important polyunsaturated fatty acid for the maintenance of normal epidermal homeostasis). The results from these studies showed that the PKC isozymes identified in the epidermal nuclear fraction differ from those identified in the cytosol and plasma membrane fractions. For instance, the reported major isozymes in the epidermal cytosol and plasma membrane fractions are PKC-α and PKC-β (Cho and Ziboh, 1994a). In contrast, the major isozymes identified in the purified nuclear fraction are nPKC-α and aPKC-ζ (**Fig 6**). Furthermore, our findings that associate the upregulation of the nPKC with epidermal hyperproliferation are consistent with reports that PKC isozymes are known to be involved in both proliferative and differentiating programs in various tissues. Interestingly, at least five (α, δ, ε, η, ζ) PKC isozymes have been described in the murine keratinocytes (Dlugosz *et al*, 1992; Denning *et al*, 1993). In particular, PKC-α isozyme was associated with keratinocyte differentiation in response to increasing concentration of extracellular calcium (Denning *et al*, 1995). Taken together, these findings underscore the diversity in the subcellular localization of the PKC isozymes in the guinea pig epidermis on the one hand, and the *in vivo* guinea pig epidermal PKC isozymes and the PKC isozymes in the murine keratinocytes on the other. Generally, cell types that overproduce some or all of the PKC isoforms proliferate more, due to their susceptibility to mitogenic effects of growth factors. For example, increased expression of PKC-βII that is specifically required by erythroleukemic cells is associated with proliferation of the cells (LaPorta and Commolli, 1994). Similarly, excess of aPKC-ζ has been reported to cause deregulation of growth in mouse fibroblasts (Berra *et al*, 1993). PKC activation in the nucleus has been observed in various cells in response to growth factors such as IL-3 (Fields *et al*, 1989), serum mitogenic factors (Lacasa *et al*, 1995), and carcinogens (LaPorta and Commolli, 1994). Whereas PKC on the one hand appears to be translocated into the nucleus from the cytosol after activation in certain cells, on the other hand there are reports that show PKC isozymes to be present in the nucleus of resting cells (Capitani *et al*, 1987; Buchner *et al*, 1992). Buchner *et al* (1992) has reported the presence of a constitutively active "membrane-inserted" form of nuclear PKC activity, which can be further stimulated by detergent treatment. In proliferating and differentiating adipocytes, aPKC-ζ was present in the nuclei of unstimulated cells (Lacasa *et al*, 1995). Stimulation of these cells with insulin or serum caused a rapid increase in nuclear PKC-ζ, suggesting that aPKC-ζ could directly phosphorylate structural and/or regulatory nuclear proteins. PKC has also been shown to be extremely important in mitogenic signaling, commencing with its dramatic activation of the downstream ERK family of proteins such as MEK and MAP-kinase (Nishida and Gotoh, 1993). Although our data presented in **Fig 8** revealed that MAP-kinase was expressed in the nuclear fraction, the level of MEK was not detectable, suggesting that MAP-kinase, and not MEK, may be the direct substrate for nuclear PKC in the guinea pig epidermis, although this possibility remains to be delineated.

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